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Abstract

Approximately 20% of the UK population wear some form of denture prosthesis, resulting in denture stomatitis in half of these individuals. *Candida albicans* is primarily attributed as the causative agent, due to its biofilm forming ability. Recently, there has been increasing evidence of *C. albicans* biofilm heterogeneity and the negative impact it can have clinically; however, this phenomenon has yet to be studied in relation to denture isolates. The aims of this study were to evaluate *C. albicans* biofilm formation of clinical denture isolates in a denture environment, and assess antimicrobial activity of common denture cleansers against these tenacious communities. *C. albicans* isolated from dentures of healthy and diseased individuals was quantified using real-time polymerase chain reaction and biofilm biomass assessed using crystal violet. Biofilm development on the denture substratum poly(methyl methacrylate), Molloplast B and Ufi-gel was determined. Biofilm formation was assessed using metabolic and biomass stains, following treatment with denture hygiene products. Although *C. albicans* was detected in greater quantities in diseased individuals, it was not associated with increased biofilm biomass. Denture substrata were shown to influence biofilm biomass, with poly(methyl methacrylate) providing the most suitable environment for *C. albicans* to reside. Of all denture hygiene products tested, Milton had the most effective antimicrobial activity, reducing biofilm biomass and viability the greatest. Overall, our results highlight the complex nature of denture related disease, and disease development cannot always be attributed to a sole cause. It is the distinct combination of various factors that ultimately determines the pathogenic outcome.

Introduction

It is estimated that 1 in 5 adults in the UK wear some form of dental prosthesis (1). Given that such a large proportion of the adult population wear a denture, then denture-related oral disease is a substantial healthcare problem. Denture stomatitis (DS) is considered the most common disease to afflict denture wearers, and has been shown to affect approximately 50% of this population (2). *Candida spp*., primarily *C. albicans*, are generally acknowledged as the principal microbial agents driving the pathogenesis of DS (3), although other species such as *C. glabrata* are frequently co-isolated (4). *C. albicans* is able to adhere to the denture surface and form complex microbial communities known as biofilms (5), surrounded by an extracellular matrix (ECM), which confers protection from antimicrobials (6, 7). These tenacious structures have been shown to adhere strongly to the denture. However, the details of such surface interactions are not yet fully understood (8, 9). Moreover, this problem is further compounded by the fact that *C. albicans* biofilms are clinically heterogeneous, and this phenotype has been shown to negatively impact patient outcomes (10-12). Whether this clinical heterogeneity plays a role in the intra-oral denture environment remains to be determined, as the influence of denture surfaces and methods of decolonisation needs to be considered.

Poly (methyl methacrylate) (PMMA) is clinically the most commonly used denture material due to low cost and easy manipulation, despite its varied topography, which positively influences microbial colonisation and biofilm development (13-15). PMMA is a rigid material that, when in close contact with the mucosa, can cause discomfort. Therefore, the demand for softer, less rigid materials is becoming increasingly popular. These include resilient soft liners such as silicone- and acrylic-based liners, which have been shown to improve patient masticatory function and reduce pain and the appearance of sore spots within the oral cavity (16, 17). However, these liners are not resistant to the colonisation of microorganisms, complicating denture hygiene even further (14, 18). A number of over-the-counter (OTC) denture cleansers are widely available, though clinical guidelines advising on the most appropriate cleansing regimen are varied and lack a comprehensive evidence base. Denture cleansing alone has demonstrated a significant reduction in microbial viability and biomass, though a reservoir of cells remain upon the surface (19). Combining

mechanical and chemical intervention can further aid the control of biofilm formation on PMMA, though again residual biofilm cells are still capable of persisting (20, 21).

The primary objective of this study was to determine whether quantitative (numbers of biofilm cells) or qualitative (biofilm phenotypic heterogeneity) factors from *C. albicans* were the primary drivers of denture stomatitis. A secondary objective was to evaluate whether the biofilm phenotype was influenced by an *in vitro* denture environment and denture hygiene treatment. Here we report that *C. albicans* isolates from dentures are quantitatively associated with disease but not biofilm heterogeneity. *In vitro* modelling revealed that a denture-related environment impacts biofilm heterogeneity, and how these respond to denture hygiene products.

Methods

Patient sampling

Samples for this study were obtained from 129 denture wearers attending the Glasgow Dental Hospital and School for routine treatment, as previously described by our group (22). Written informed consent was obtained from all participants. Ethical approval was granted by the West of Scotland Research Ethics Service (12/WS/0121) for the recruitment of participants to this study, where a clinician was responsible for the collection of clinical samples. The presence or absence of DS was assessed by the clinician, and those patients with DS were grouped according to Newton's classification (23). The following scores were applied: 0 for healthy mucosa, 1 for pin-point hyperaemic lesions (localised erythema); 2 for diffuse erythema (generalised simple inflammation); and 3 for hyperplastic granular surface (inflammatory papillary hyperplasia). Patients were excluded from this study if they had been receiving antimicrobial treatment or using prescription mouthwashes within six months prior to sampling. Dentures were removed from the patient's mouth and placed in sterile bags (Fisher Scientific) filled with 50 ml phosphate buffered saline ([PBS, Sigma-Aldrich), and placed in a sonic bath (Ultrawave) for 5 min to remove the biofilm from the surface (24). The denture sonicate was centrifuged for 10 min at 3700 x *g* [6000 r.p.m.], and the plaque pellet resuspended in 2 ml of RNAlater® (Qiagen) and stored at -80 °C.

Molecular quantification of Candida in clinical samples

The presence of *Candida* spp. isolated from healthy (n=81) and DS (n=48) patients was assessed by quantitative PCR (qPCR) by amplifying the *Candida* specific 18S gene. DNA was extracted using the QIAamp® DNA mini extraction kit (Qiagen) according to manufacturer's instructions, with a minor modification to include a mechanical disruption step with sterile acid-washed glass beads of 0.5 mm diameter (Thistle Scientific). This was achieved by beating the samples for 3 × 30 s on a Mini-BeadBeater (Sigma-Aldrich), while intermittently being placed with intermittent

cooling. DNA quality and quantity was quantified using the NanoDrop® spectrophotometer (ThermoScientific).

To quantify *Candida* from each sample, 1 µl of DNA was added to a mastermix containing SYBR® GreenER™ (Life Technologies), UV-treated RNase-free water and 18S forward/reverse primers (10 µM, Forward - CTCGTAGTTGAACCTTGGGC; Reverse - GGCCTGCTTTGAACACTCTA (25)), as per manufacturer's instructions. The thermal profile used consisted of 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95°C for 3 s and 60 °C for 30 s using a StepOne Plus real-time PCR unit (Applied Biosciences). Cycle threshold (Ct) values were used to approximate the number of corresponding colony forming equivalents (CFE's) based on standard curves (Supplementary Figure 1).

Standardised Candida albicans biofilm assessment

To detect and identify the *Candida* species present, 100 µl of the denture sonicate was spread across Colorex *Candida* agar (E & O labs) and incubated at 30 °C for 72 h. All clinical isolates obtained during this study were stored in Microbank® vials (Pro-Lab Diagnostics, Cheshire, UK) at −80 °C, until required. Isolates identified as *C. albicans* from healthy (n=31) and DS (n=37) patients were propagated in yeast peptone dextrose (YPD) medium (Sigma-Aldrich), incubated at 30 °C, and vortexed 150 r.p.m. for 18 h. Following incubation, cultures were washed twice by centrifugation, standardised, and grown statically at 37 °C for 24 h in 96 well flat-bottomed polystyrene plates (Corning Incorporated), and assessed for biofilm formation as previously described (10, 26). Each isolate was tested in triplicate and negative controls containing no *C. albicans* were also included. Following incubation, biofilms were carefully washed twice with PBS to remove any non-adherent cells, before biomass was assessed using the crystal violet (CV) assay, as described previously (27). Absorbance was read spectrophotometrically at 570 nm and isolates were grouped based on their level of biomass distribution. Isolates that fell below the 1st quartile (Q_1, OD_{570} = 0.382) were classed as low biofilm formers (LBF) and strains with a biomass greater than the 3rd quartile (Q_3, OD_{570} = 1.192) were deemed as high biofilm formers (HBF), as described previously (10, 11).

Assessment of biofilm formation upon different denture materials

Given the artificial nature of standardised *C. albicans* biofilm testing in RPMI-1640 medium and polystyrene, we next wanted to assess how selected isolates behaved in an environment representative of media and substratum, namely artificial saliva (AS) and denture materials. Two clinical isolates (n=1 for LBF and n=1 for HBF) and one laboratory strain (SC5314) were selected for assessing their biofilm formation upon various denture materials. Isolates were standardised to 10^7 cells/ml in AS (porcine stomach mucins 0.25% (w/v), sodium chloride 0.35% (w/v), potassium chloride 0.02 (w/v), calcium chloride dihydrate 0.02% (w/v), yeast extract 0.2% (w/v), lab lemco powder 0.1% (w/v), proteose peptone 0.5% (w/v) in double distilled water (ddH_2O). Urea was then added independently to a final concentration of 0.05% (v/v), as previously described (28), and 1 ml added to each well of a 24 well flat-bottomed polystyrene plate (Corning Incorporated) containing the following denture substratum 'in house' manufactured discs (13 mm × 1 mm): PMMA MOLLO (Detax dental) and UFI (Voco GmbH) soft liners. Biofilms were incubated at 37 °C for 4 h and 24 h before washing twice with PBS. As the biofilms were grown on the denture material discs, CV could not be used to accurately quantify biomass. Therefore, qPCR was used instead. Biofilms were sonicated at 35 kHz for 10 min in 1 ml PBS to remove the biomass (29). Samples were centrifuged for 10 min at 10000 x *g,* the supernatant discarded and DNA extracted, as previously described. qPCR was carried out and *C. albicans* cells were quantified using the 18S primers, as previously described.

Antimicrobial treatment of Candida albicans biofilms

Five *C. albicans* clinical isolates defined as HBF were selected to test a range of OTC denture cleansers (Steradent, Milton and Poligrip) against pre-formed biofilms. Isolates were grown in a 96-well flat-bottomed plate for 4 h and 24 h. Two hundred µl of these solutions were added to each biofilm and treated, as per defined by the manufacturer (10 min for Steradent; 15 min for Milton; 3 min for Poligrip). Untreated controls were included for comparison. Following treatment, biofilms were washed with PBS and standardised XTT and CV assays were carried out to assess biofilm

metabolic activity and biomass, respectively, as described previously (11, 26). Scanning electron microscopy (SEM) was performed on the *C. albicans* biofilms grown on PMMA discs to visualise the effects of denture cleansing. Following 4 h and 24 h biofilm development, cells were treated with the various denture cleansers before carefully washed with PBS, fixed, processed and imaged, as previously described (30).

Statistical analysis

Graph production, data distribution and statistical analysis were performed using GraphPad Prism (version 5.01). Data was first assessed to see if it configured to a normal distribution and was log transformed where necessary. Student t-tests were used to compare candidal loads of healthy and diseased patients. A one-way ANOVA with Bonferroni correction was used to measure statistical differences between biofilms grown on different denture materials and antimicrobial viability testing of the isolates. A Kruskal-Wallis test with Dunn's post-test was used to measure differences in biofilm biomass following different denture cleansers.

Results

Candida burden differs in healthy and DS denture wearers

The candidal burden colonising the denture surface differed significantly between healthy and DS patients (Fig. 1(a); $p<0.001$). Nonetheless, significant overlap was seen between both groups, emphasising the variable nature of candidal biofilm formation. Next, the biofilm forming ability of *C. albicans* denture isolates was assessed to evaluate biofilm heterogeneity (Fig. 1b). Isolates differentially formed biofilms and were classed as either LBF (Q_1, OD_{570} = 0.382) or HBF (Q_3, OD_{570} = 1.192). Isolates in between these groups were classed as intermediate biofilm formers. Optical densities for healthy patients ranged from 0.09 to 2.81 and from 0.1 to 2.06 for DS patients, across the 68 isolates tested. No statistical difference was observed between healthy and DS patients ($p>0.05$), with biomass regarded as highly variable. This confirms that *C. albicans* biofilm formation is heterogeneous within both groups.

Biofilm biomass and viability is impacted by media and denture substratum

The impact of denture substratum upon *C. albicans* biofilm formation was assessed next (Fig. 2). At 24 h of biofilm formation, PMMA was the denture material with the most significant *C. albicans* burden (4.21×10^8 CFE), this being 2.8 times and 4.1 times greater than those of MOLLO ($p<0.05$) and UFI ($p<0.05$), respectively. Unsurprisingly, *C. albicans* biomass was significantly more abundant in 24 h biofilms than in their 4 h counterparts for each denture substratum; PMMA ($p<0.001$), MOLLO ($p<0.05$) and UFI ($p<0.001$). At early stages of biofilm development (4 h), no significant candidal burden was observed between the three substrata ($p>0.05$). When biofilm heterogeneity was assessed upon denture substrata, no significant differences in *C. albicans* burden where found between LBF and HBF (data not shown).

Treatment of C. albicans biofilms with antimicrobial denture cleansers

Finally, three OTC denture cleansers were selected to assess their antifungal activity against *C. albicans* denture biofilms formed by HBF. Milton was the most effective cleanser against early biofilms (4 h), reducing biomass by 55%, compared to the untreated control ($p<0.05$), as shown in Fig. 3(a). Poligrip was the second most active agent, reducing biomass by 28%. However, Steradent led to an increase in the denture biomass by 9%. When mature biofilms were considered (24 h), no significant differences were observed when comparing the denture cleanser treatments to control biofilms. Unsurprisingly, biomass increased between 4 and 24 h by 3.3, 2.8, 5.2 and 5 times for the untreated, Steradent, Milton and Poligrip, respectively.

As Milton was the most effective cleanser at disturbing biofilm biomass, it was predicted that it would also be the most superior at reducing cellular viability. In this study, we have shown that Milton reduces metabolic activity to 17% and 56% in 4 h ($p<0.001$) and 24 h ($p<0.05$) biofilms, respectively (Fig. 3(b)). The cellular viability at 4 h was less than that at 24 h, which is most likely due to early biofilms not being as complex as their 24 h counterparts, meaning antimicrobials are able to penetrate more easily, elicit their activity and reduce viability more readily than the can in mature biofilms. Poligrip had little impact on biofilm development, with 90% viable cells remaining following treatment of 4 h biofilms, and 78% at 24 h. Interestingly, steradent was shown to have the greatest activity against mature biofilms, reducing cell viability to 67% at 24 h, compared to no reduction at 4 h ($p<0.001$). This being said, all denture-cleansing regimens were unable to reduced 24 h biofilm viability to less that 50%.

Ultrastructural changes of the treated denture biofilms are illustrated in Fig. 3(c). Steradent-treated early biofilms exhibited minimal changes in appearance compared to the untreated control. However, at 24 h the biofilms were visually distinct, with a fibrous residue evident along the elongated hyphae. This may be a remnant of the denture cleanser retained within the biofilm as this was also observed in Milton and Poligrip mature biofilms. Interestingly, at 4 h, the hyphal cells appeared to have become embedded with the denture acrylic, which may be due to the cleansers becoming incorporated within the material and changing the surface topography, as suggested elsewhere (31, 32).

Discussion

Despite the vast improvements in oral health over the last 50 years, there still remains a large proportion of the adult population that rely on some form of denture prosthesis. Wearing a denture can be an immensely uncomfortable experience (33), with DS being one of the primary complaints affecting half of denture wearers at some point in their lives (3). *C. albicans* colonisation and subsequent infection of mucosa is the principal cause of DS. Here we report that, although biofilm heterogeneity impacts overall effective denture cleansing, the quantity of candidal yeasts is the main driver of denture stomatitis, not the biofilm phenotype nor denture substratum, a finding supported by previous researchers (34).

It is interesting that heterogeneity is apparent amongst clinical isolates attached to dentures, and this phenomenon may play a yet undefined role in DS. Clinically, the concept that biofilm heterogeneity may play an important role in determining morbidity and mortality levels is worthy of investigation in the context of the oral cavity. We have recently reported an association with this phenotype and mortality in a Scottish candidaemia cohort (10), so therefore we undertook an analogous investigation in a cohort of denture patients. Interestingly, although biofilm heterogeneity was observed using established models, an association with patient outcomes was not shown. Our inability to demonstrate cause and effect may be explained by the fact that this is a relatively mild disease in comparison to candidaemia from a severely immunocompromised group. Nevertheless, a positive correlation between the absolute candidal numbers and disease was observed, suggesting that the physical interaction between large numbers of yeasts and hyphae on the denture surface was more important than the phenotype *per se*. This is likely influenced by the diverse microbiota in this environment, which we have recently described (22). Indeed, there is increasing evidence that *Candida* and bacteria form polymicrobial biofilms, and that some bacterial species common to the oral cavity can enhance the pathogenicity of *C. albicans* (35-38), and that the presence of specific oral bacteria is enough to transform a *C. albicans* LBF into a HBF (39). We have also reported a significant association between lactobacilli and yeasts in this context, and this may be more important than *C. albicans* alone, as is the perceived paradigm for this DS. Therefore, the local environment of the denture

may play an important role in determining how denture related stomatitis ensues. Studies in mice infected with HBF strains of *C. albicans* were shown to survive significantly longer than those infected with LBF strains, suggesting a competitive advantage in the host environment (40).

The microbiology of dentures is dictated by substrata and the surrounding host environment. Changing this environment can vastly influence *C. albicans* biofilm formation and survival. Initial adhesion of microbes to dentures is key to their pathogenicity, yet denture substrata are highly variable, which may support differential adherence and biofilm formation. PMMA is classically used as a denture substratum, though its chemistry and topography create a preferential surface for biofilm formation (41). Alternative soft liner materials are increasing in popularity as they are softer against the palate as well as providing improved masticatory function over traditional denture materials (16). Studies comparing microbial growth on hard and soft denture liners have shown conflicting results, as one study comparing *Candida* biofilm growth on various soft denture liners found no significant differences in the quantity of cells recovered between materials (42). Our study however, found that, although there was no significant difference in microbial colonisation during early biofilm formation, PMMA became the most prone substratum to biofilm formation at later stages of development, regardless of the isolate's phenotype. Interestingly, analysis of biofilms forming on PMMA and soft liners at 1, 7 and 14 days, found no significant differences in adhesion at 24 h, yet colonisation on hard acrylic was observed to be lower at all time points (43). A possible explanation as to why we are seeing higher numbers on PMMA is because soft liners are even more porous in nature than hard acrylics (44) and thus higher numbers of microbes may become imbedded in these cracks and crevices. Therefore, even a powerful method such as sonication may not be sufficient enough to completely dislodge the microorganisms hidden within these soft liners. To the authors' knowledge, this is the first study to use molecular methods such as qPCR to quantify biofilms on denture acrylics, in order to give more accurate quantitative microbial counts. However, the vast majority of studies in this area have relied on culturing and staining techniques for quantification, and thus the methods used may account for the differences seen across studies (42, 43, 45).

Currently, there is no universal method for cleaning dentures, where it is common to find the recommended method varies from dentist to dentist. There are numerous OTC denture cleansers available. Therefore, we selected three of the most popular brands - Steradent, Milton and Poligrip - to assess their antifungal activity. Milton was the most effective treatment at reducing biomass and cell viability on early (4 h) biofilms. Yet, it did not completely eradicate the biofilm. Such results are interesting given previous findings showing that this treatment completely inhibited biofilm formation at 4 h (29). Methodological constraints due to conventional c.f.u. quantification may explain this, since it has been recently demonstrated counting c.f.u. can give false negative results. By using live/dead qPCR on the same experiment, ~ 1 x 10^5 cells/ml viable cells were detected, where c.f.u. counting detected no live cells (21). Moreover, as the biofilms matured, Milton lost its effect, most likely because of the thicker biofilm, which would be more difficult to penetrate and disrupt. Yet, previous experiments have shown that denture cleansers and antimicrobial mouthwashes can significantly reduce the viability of mature *C. albicans* denture isolates (19, 46). These studies do not confirm the biofilm forming capacity of the assayed isolates, whereas our study used known HBFs, which could explain why our isolates were more resistant to treatment, a phenomenon reported elsewhere with these phenotypes (11). Moreover, previous studies have shown that *C. albicans* is not completely eradicated from the denture, even when using various denture cleansing treatments (29). Our collection of SEM images supports this as they show that *C. albicans* cells remain on the denture acrylic irrespective of treatment. We cannot guarantee that all these cells are dead and therefore it is likely that some are still alive and could begin to recolonise the denture when placed back into the mouth. Furthermore, full penetration of the biofilms is unlikely due to the protective effects against antimicrobials, such as efflux pumps and extracellular glucans, provided by the ECM". (30, 47).

In conclusion, it was demonstrated that *C. albicans* isolates reside on dentures on DS affected individuals in higher numbers. These isolates demonstrated heterogeneity in their biofilm forming abilities. However, HBFs were not more common to DS sufferers. Therefore, although the particular strain isolated may strongly influence the development of disease, other factors are also involved. The surrounding environment has a strong influence as we demonstrated that some

materials are better at supporting *C. albicans* biofilm growth. Furthermore, antimicrobial denture cleansing treatments do not fully eradicate HBF *C. albicans* isolates, leaving behind live cells, which can disperse and recolonise. Therefore, in terms of future studies investigating *C. albicans* biofilms and denture related disease, it has to be recognised that various factors influence disease and that it is the culmination of these that determines the pathogenic outcome.

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Abbreviations

AS: artificial saliva; CFE: colony forming equivalent; ddH_2O: double distilled water; DS: denture stomatitis; ECM: extracellular matrix; HBF: high biofilm formers; LBF: low biofilm formers; MOLLO: molloplast B; OTC: over-the-counter; PMMA: poly (methyl methacrylate); qPCR: quantitative polymerase chain reaction; SEM: scanning electron microscopy; UFI: ufi-gel.

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Figure 1: **Quantification of *Candida* species and biomass from clinical isolates.** The denture sonicates of healthy or denture stomatitis (DS) individuals were screened for *Candida* presence **(A)**. Both horizontal lines represent the mean of each group, with the asterisks representing significant differences (***$p<0.001$). Low biofilm formers (LBF) and high biofilm formers (HBF) isolates were defined, respectively, by the lower and upper quartiles, as defined by the dotted lines **(B)**.

Figure 2: **Impact of the tested denture substratum on *Candida albicans* biofilm formation.** *C. albicans* SC5314, one low biofilm former and one high biofilm former were grown as biofilms on PMMA, MOLLO and UFI denture materials, to assess biofilm formation at 4 h and 24 h. Data represents mean ± SD of three isolates combined together. 24 h data was compared to 4 h counterparts ($^{\#}p<0.05$, $^{\#\#\#}p<0.001$). Significant differences were also observed between different denture substratum at 24 h (*$p<0.05$).

Figure 3: **Assessing antimicrobial denture cleansers on *Candida albicans* biofilm formation.** The effects of three over-the-counter (OTC) denture cleansers on *C. albicans* biofilm formation were assessed by biomass **(A)** and viability **(B)** at 4 h and 24 h. All images are shown at 1000× magnifications **(C)**. Scale bars represent 20 μm. Data represents mean ± SD. Significant differences were observed when comparing each cleanser at 4 h to their 24 h counterparts ($^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$), as well as comparing cleansers to one another (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).